ZOOLOGICAL SCIENCE 10: 117-125 (1993)

Reproductive Characteristics of Precociously Mature Triploid Male Masu Salmon, *Oncorhynchus masou*

Masaru Nakamura[1], Fumio Tsuchiya[2], Masao Iwahashi[2] and Yoshitaka Nagahama[3]

[1]*Department of Biology, Faculty of Medicine, Teikyo University, Hachioji, Tokyo 192-03,*
[2]*Niigata Prefecture Inland Water Fisheries Experimental Station, Koide-cho, Kitauonumagun, 946, and* [3]*Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki, 444 Japan*

ABSTRACT—In order to clarify the reproductive characteristics of triploid males, changes in serum steroid hormone levels and testicular development in precociously mature triploid males of masu salmon, *Oncorhynchus masou*, were examined during sexual maturation. Testicular development in triploids differs from that in diploids. The gonadosomatic index (GSI) in triploids in August was high (3.9%), similar to that in diploids (3.0%). Although germ cells at all stages of spermatogenesis were observed in the testes of diploids, the testes of triploids consisted of many cysts of primary spermatocytes and some degenerating germ cells. In the spawning season in October, GSI in triploids dropped suddenly (1.0%), though GSI in diploids increased (4.8%). At that time, a large number of degenerating figures of spermatogenic germ cells were found in the testes of triploids. Very few sperm were seen in the sperm fluid. However, most of the sperm cells revealed abnormalities in morphology, such as two tails, two heads, and heads of various sizes. Changes in serum testosterone (T), 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOH) in triploid males were similar to those in mature diploid males. T and 11-KT levels were high in August and October, then they dropped in November after the spawning season. 17α,20β-DiOH levels in both triploid and diploid males were only high in October during the spawning season. From these results, it seems likely that the cause of abnormality of spermatogenesis in triploid males is mechanical difficulty involved in chromosome separation at meiosis I due to triploidy *per se*, rather than the reduced levels of testicular steroid hormones.

INTRODUCTION

Recently it has become possible to manipulate chromosome sets by means of physical or chemical treatment of eggs at an early developmental stage [7, 12]. This technique of chromosome set manipulation is expected to be applicable to practical fish culture in a variety of ways, such as sex determination or improvement of breed [7]. Triploid fish can be induced by retaining the second polar body of eggs just after fertilization. Triploid fish are thought to become sterile as a consequence of having an odd number of chromosome sets. This sterile triploidy may bring about benefits to practical fish culture for the reason that sterile fish avoid the muscle deterioration and death which accompany sexual maturation [10].

Induction of triploidy has been attempted in several fishes so far. However, characteristics of sexually mature triploids depend on sex and species. In a previous paper, we reported the gonadal development and changes of steroid hormones in triploid female rainbow trout, *Oncorhynchus mykiss* [6]. In the present study, we deal with testicular development and changes in steroid hormones in the triploid male masu salmon, *Oncorhynchus masou*.

Accepted November 4, 1992
Received September 18, 1992

MATERIALS AND METHODS

Land-locked masu salmon (Yamame) used in

the present study were obtained from the Niigata Prefecture Inland Water Fisheries Experimental Station. They have been cultivated systematically in ponds. Mature eggs and sperm were obtained from randomely selected 2-year-old females and males. To induce triploidy, just after artificial insemination, eggs were immersed in warm water at 27–31°C for 10–15 min. After this temperature shock, eggs were removed and put into water at about 12°C. Fry were fed on the regular diet for salmon culture from the first feeding.

Precociously mature triploid males (10 to 13 month-old) were autopsied 3 times, August 30, October 22, and November 25, 1986. For each sample, body weight, body size, and testicular weight were measured. To confirm triploidy, a blood smear from each individual was prepared. After staining with 1% Giemsa solution, long diameters of erythrocytes were measured. Testes of triploids were fixed in Karnovsky's solution for 2 hr. Next, they were postfixed in 1% OsO_4 buffered to pH 7.4 with 0.1 M cacodylate buffer for 2 hr. After dehydration, they were embedded in epoxy resin. For histological observations, one micrometer sections were stained with 1% toluidine blue in 0.1 M phosphate buffer.

For the investigation of sperm, sperm fluid was collected in the October spawning season. Smears of sperm fluids were prepared and stained with Delafield's hematoxilin.

For the measurement of steroid hormone levels in serum, blood from each fish was collected by cutting the caudal region. The serum was separated by centrifugation, frozen at −20°C, and stored until use. Serum levels of testosterone (T), 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOH) were measured by radioimmunoassay [3, 8, 13, 14].

Precociously mature diploid males were sacrificed in the same manner and at the same time, and they served as the control.

Data were analyzed using Student's *t*-test (erythrocyte diameter) and two-way analysis of variance (ANOVA, GSI and body weight), accepted at $P<0.05$.

RESULTS

Diameter of erythrocytes

Erythrocytes of diploid and triploid males are pictured in Figs. 1A and 1B, respectively. Diameter of erythrocytes of triploids is 19.22 ± 0.81 μm. This value is significantly larger ($P<0.01$) than that of diploids (14.62 ± 0.95 μm). Triploids were distinguished from diploids by the size difference in erythrocytes.

Growth and external view of fish

Body weight and total length of triploid and diploid males is shown in Table 1. Diploid and

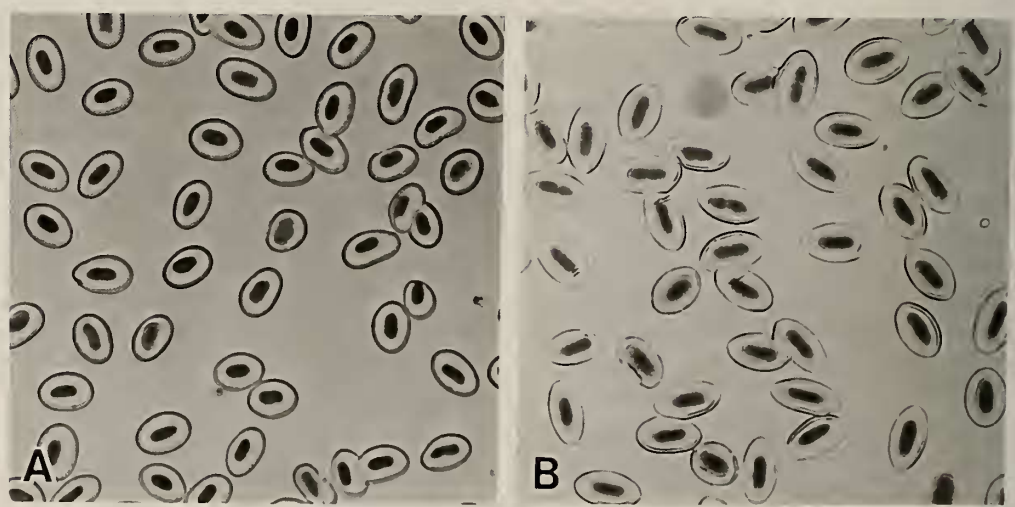

FIG. 1. Erythrocytes of diploid (A) and triploid (B) masu salmon. ×610.

TABLE 1. Comparison of body weight (B.W.) and total length (T.L.) of triploid and diploid males in each sampling time

		Aug. 30	Oct. 22	Nov. 25
Triploid	B.W. (g)	36.7±3.1	48.1±2.3	51.3±3.0
	T.L. (cm)	14.5±0.2	16.7±0.4	17.6±0.3
Diproid	B.W. (g)	32.7±2.5	51.3±3.7	55.4±5.0
	T.L. (cm)	14.7±0.3	17.4±0.3	18.0±0.5

Each value is the mean±SE.

triploid males grew similarly and showed no differences in body weight and total length at any sampling time.

Triploid individuals were not different from diploids in external view (Fig. 2). Male secondary sex characters such as dark color on the skin of body and each fin appeared normally in triploids in the October spawning season, similar to diploid males. About 70% of triploid males matured precociously one year after fertilization. This value was almost the same as that of diploid males.

Changes in gonadosomatic indices (GSI=(gonad weight/body weight)×100)

Changes in GSI in diploid and triploid males are shown in Figure 3. Changes in GSI in triploids differ from those in diploids. GSI in triploids was high, 3.9±0.6%, in August, as was GSI in diploids (3.0±0.9%). In October, GSI in diploids increased further to 4.8±0.8%. In contrast, GSI in triploids dropped markedly to 1.0±0.7%, even in the spawning season ($P<0.01$). GSI in diploids dropped rapidly (1.5±0.2%) in November after the spawning season. GSI in triploids dropped even more (0.2±0.01%) ($P<0.01$).

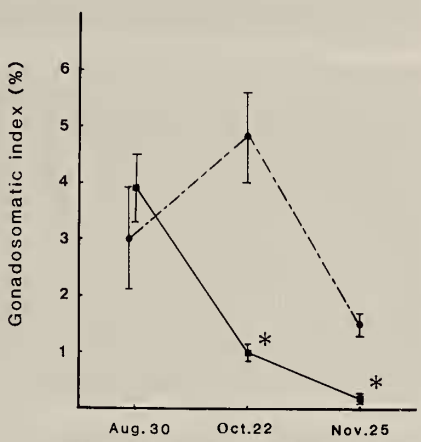

FIG. 3. Changes in the gonadosomatic index (mean± SE) of diploid (---●---) and triploid (—■—) masu salmon. *Significantly different from diploid ($P<0.01$).

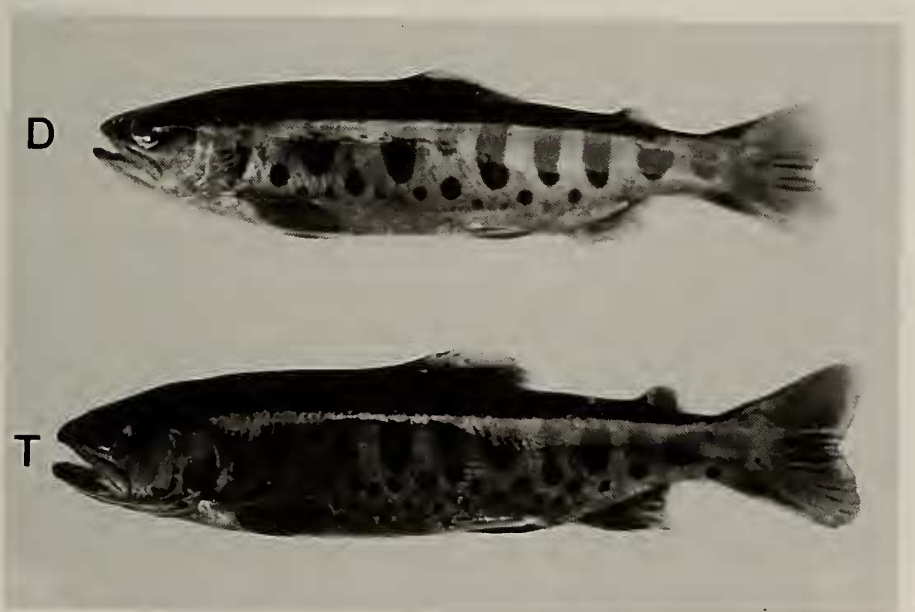

FIG. 2. The secondary sex character of dark skin appears in both diploid (D) and triploid (T) male masu salmon.

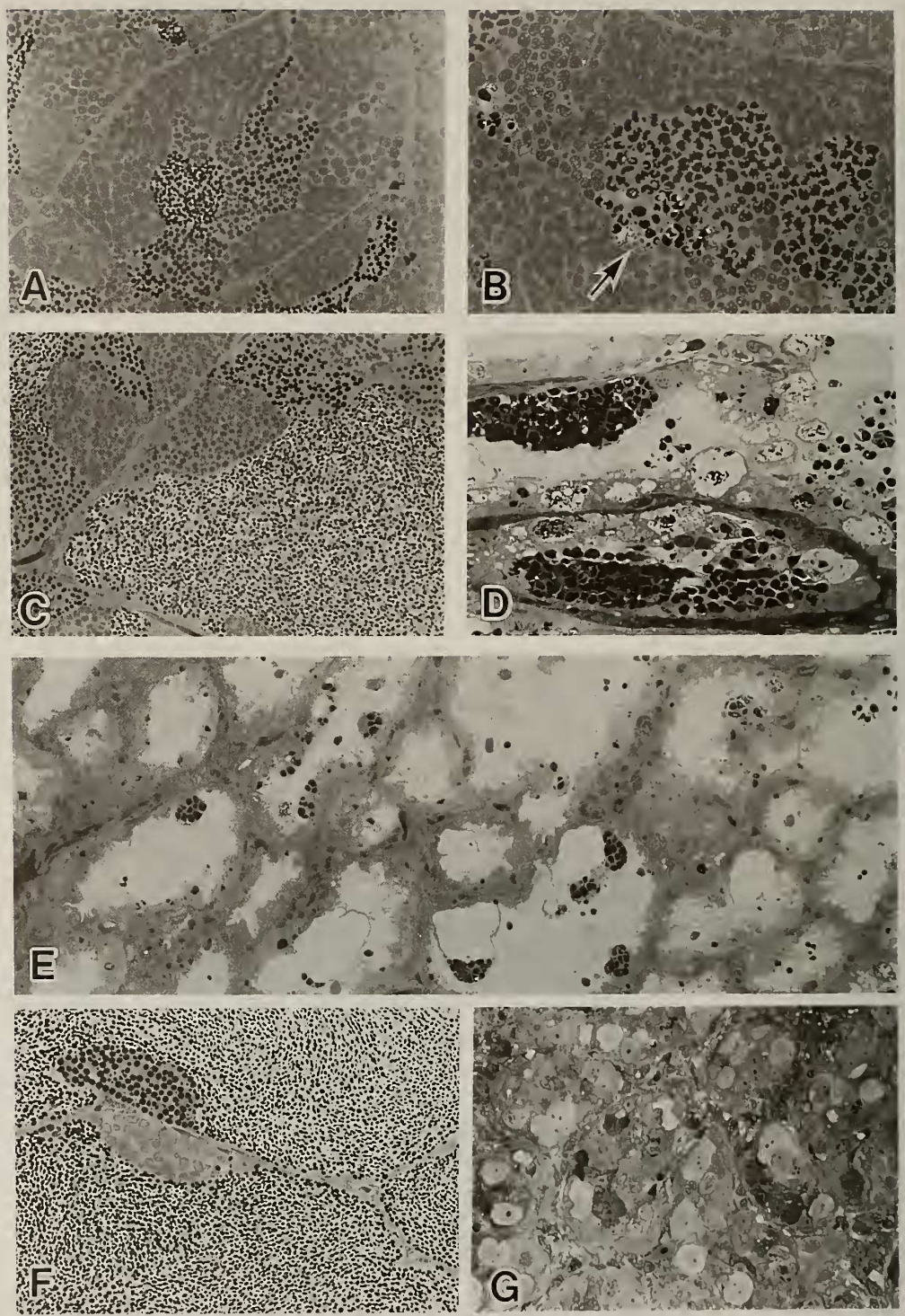

FIG. 4. Photomicrographs of testes from diploid and triploid male masu salmon. Diploid testes in August (A), October (C) and November (F). Triploid testes in August (B), October (D and E) and November (G). ×590.

Testes

Cysts of spermatogenic germ cells at various stages were contained in the testes of diploids in August (Fig. 4A). A small amount of matured sperm had already been seen in the central region of the lumen. Active spermatogenesis was also seen in the testes of triploids (Fig. 4B). However, spermatogenic germ cells consisted mainly of primary spermatocytes (Fig. 4B). Secondary spermatocytes, spermatids, and sperm were not observed in triploids, although some clusters of degenerating spermatocytes were observed in the lumen (Fig. 4B). The testes of triploids in the spawning season in October were noticeably different in size and color from those of diploids (Fig. 5). Testes of diploids were white in color and were packed with matured sperm (Fig. 5). In contrast, those of triploids were semitranslucent. Sperm fluid filled the efferent ducts. The testes of triploids (Figs. 4D and 4E) were also extremely different in histology from those of diploids (Fig. 4C). The amount of spermatogenic germ cells was markedly less in triploids (Figs. 4D and 4E) than in diploids (Fig. 4C). Moreover, these germ cells revealed necrosis. Some necrotic cells were ingested by hypertrophied Sertoli cells lining the inner wall of the lumen (Fig. 4D). Although some sperm were recognized in sperm fluid in triploids (Fig. 6B), they were fewer in number and noticeably different in morphology from those of diploids (Fig. 6A). Sperm of triploids were morphologically abnormal, such as having two heads, two tails, or various sizes of heads. Large amounts of sperm still remained in the testes of diploids in November (Fig. 4F). In the testes of triploids, the inner wall of the lobules contained only some spermatogonia (Fig. 4G).

Changes in serum steroid hormone levels

Changes in T, 11-KT, and 17α,20β-diOH levels in triploids and diploids are shown in Figure 7A-7C. High levels of serum T (diploid, 8.0 ng/ml; triploid, 7.4 ng/ml) and 11-KT (diploid, 5.7 ng/ml; triploid 5.0 ng/ml) were observed when the two groups of males were compared in August. Levels of these steroids rose further in October (diploid, T 37.0 ng/ml and 11-KT 48.1 ng/ml; triploid, T 30.8 ng/ml and 11-KT 33.3 ng/ml). T and 11-KT levels rapidly decreased in November after the breeding season in both diploid (T 0.4 ng/ml and 11-KT, not detectable) and triploid (T 0.5 ng/ml and 11-KT 0.4 ng/ml). Serum 17α,20β-diOH was not detectable in either diploids or triploids in

FIG. 5. Testes from diploid (D) and triploid (T) male masu salmon in October.

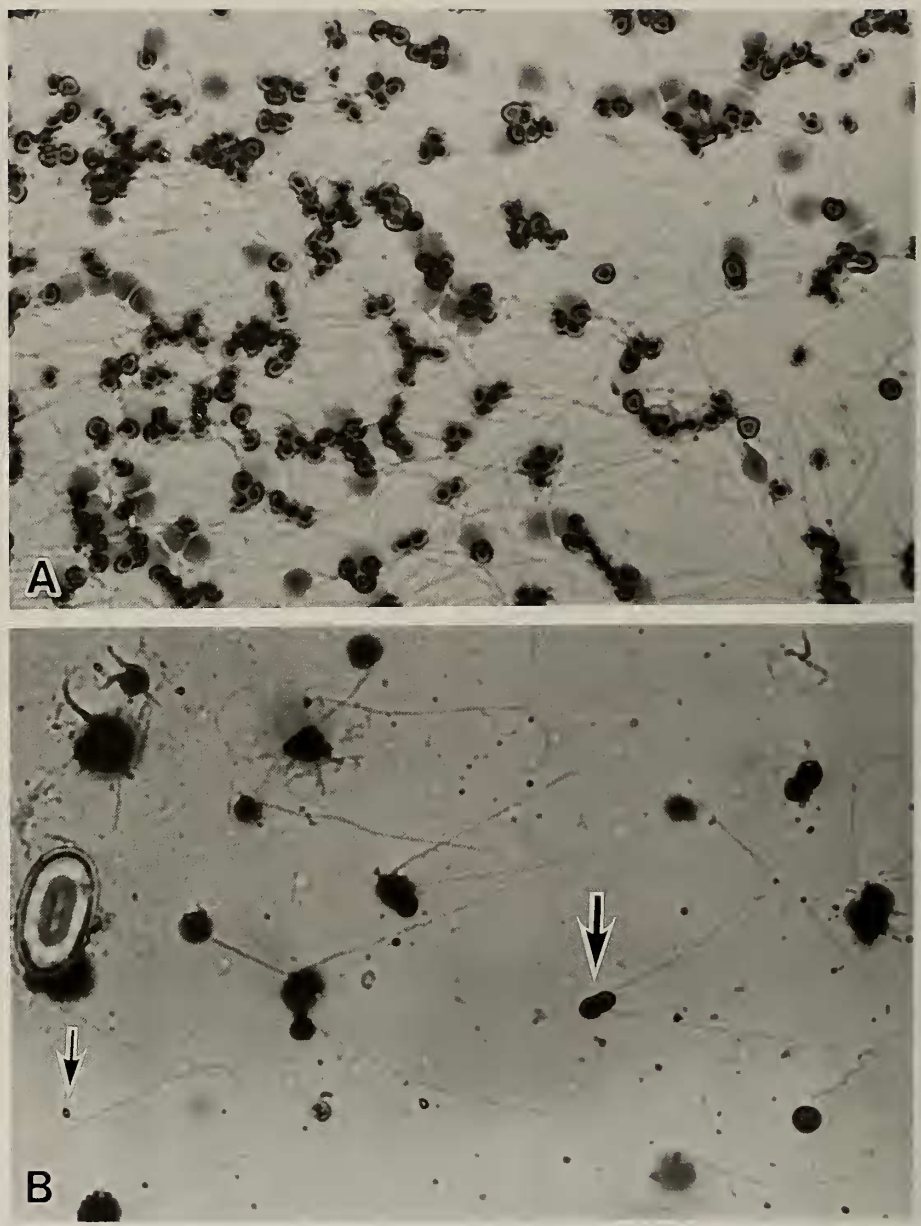

FIG. 6. Photomicrograph of sperm from diploid (A) and triploid (B) masu salmon. Small head (small arrow) and, two heads and two tails sperm (large arrow) are seen. ×1040.

August. A sharp increase of this steroid occurred in October in diploids (8.7 ng/ml) and triploids (15.9 ng/ml). 17α,20β-DiOH dropped in November (diploid 0.7 ng/ml and triploid 0.2 ng/ml).

DISCUSSION

Morphological characteristics of testicular development in triploid males have been reported in several fishes so far. In most cases, abnormalities of spermatogenesis were observed. Despite well-

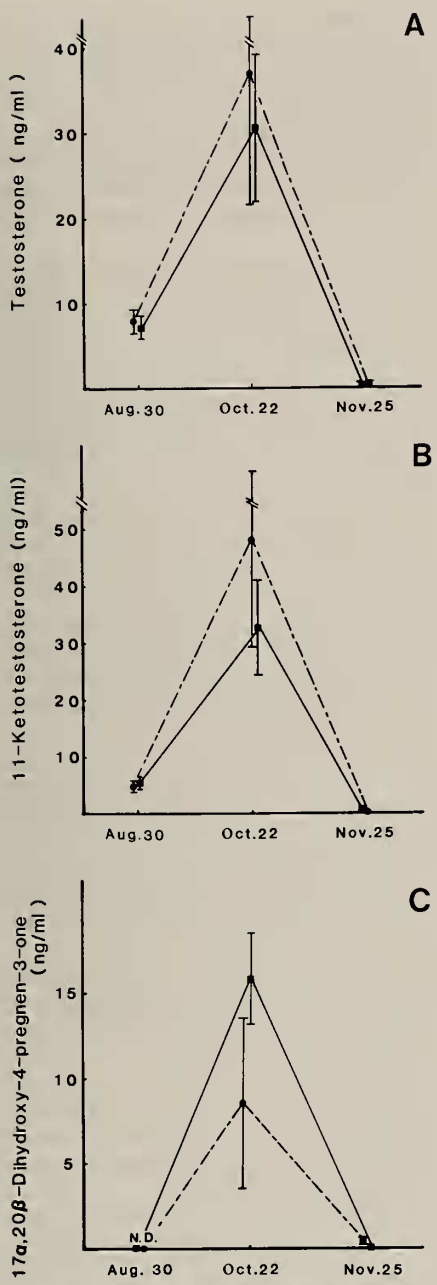

FIG. 7. Changes in serum testosterone (A), 11-ketotestosterone (B) and 17α,20β-dihydroxy-4-pregnen-3-one (C) of diploid (---●---) and triploid (—■—) male masu salmon.

developed testes, matured spermatozoa were not produced in channel catfish (*Ictalurus punctatus*) [17], nor in Atlantic salmon (*Salmo salar*) [2] or in loach (*Misgurnus anguillicaudatus*) [11]. A few sperm were formed in the testes of some species such as Biwa gudgeon (*Gnathopogon caerulescens*) [16], rainbow trout (*Oncorhynchus mykiss*) [4], and Pacific salmonids [1]. The cause of the inability to produce sperm normally in triploids is thought to be the disruption of meiosis due to the odd number of chromosome sets [12].

In the present study, it is clearly shown that the process of testicular development in triploid males is remarkably different from that of diploids. Despite the spawning season, GSI in triploids was markedly low. Sperm in triploids was less in amount and contained cells with morphological abnormalities, such as two heads, two tails, or different sized heads. In addition, secondary spermatocytes and spermatids were not found, though a large number of primary spermatocytes were formed. These facts indicate that the transformation from primary spermatocytes to secondary spermatocytes was suspended. Thus, it seems likely that the large amount of degenerating germ cells that appeared in the testes of triploids in October originated from the primary spermatocytes which failed to be transformed into secondary spermatocytes.

A few papers have dealt with the relationship between testicular development and steroid hormones in triploid fishes [1, 4, 5]. In triploid males, blood steroid hormone levels have been found to be high, just as is the case for mature diploid males. In the triploid males of masu salmon, changes of serum T, 11-KT and 17α,20β-diOH showed no significant differences in comparison with those of mature diploid males. From these results, it is concluded that the production of steroid in triploid males occurs similarly to those in diploid males, even though the development of germ cells is hindered during the process of spermatogenesis. Thus, it is presumed that abnormalities of testicular development in triploids are related to mechanical difficulties involved in chromosome separation at meiosis I due to triploidy *per se*, rather than the reduced level of steroid hormones.

On the other hand, production of steroid hormones and ovarian development in triploid females differs among species. Blood steroid hor-

mone levels in female rainbow trout [4, 6] and female masu salmon (Nakamura *et al.*, unpublished data) were extremely low, even in the spawning season. The ovaries of these female triploids were composed of numerous cysts containing many small oocytes and degenerating oocytes. In contrast, triploid female of tilapia [9] revealed high levels of steroid hormones and had well-developed yolky oocytes in their ovaries. Thus, it is still not known why the reproductive characteristics in triploid fish are different between males and females and among species.

Serum 17α,20β-diOH levels in triploid males increased only in the spawning season. Changes in this steroid are similar to those of diploid males in salmonids [9, 18], but there are no reports on the production of 17α,20β-diOH in triploid males of other species. This steroid is thought to be involved in spermiation [13]. 20β-Hydroxysteroid dehydrogenase (20β-HSD), which is the enzyme essential for the conversion from 17α-hydroxy progesterone to 17α,20β-diOH, was found to be localized on sperm [14]. Thus, it is interesting to note that 17α,20β-diOH production in triploids was as high as it was in diploids, though the amount of sperm in the testes of triploids was reduced. The testes of triploid males may provide a suitable material for the study of 17α,20β-diOH production.

ACKNOWLEDGMENTS

We would like to thank Dr. J. Specker, Department of Zoology, The University of Rhode Island, for reading the manuscript.

REFERENCES

1 Benfy TJ, Dye HM, Solar II, Donaldson EM (1988) The reproductive endocrinology of triploid Pacific salmonids. Western Regional Conference on Comparative Endocrinology p 30

2 Benfy TJ, Shutterlin AM (1984) Growth and gonadal development in triploid landlocked Atlantic salmon (*Salmo salar*). Can J Fish Aquat Sci 41:1387–1392

3 Kagawa H, Young G, Adachi S, Nagahama Y (1982) Estradiol-17β production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicle: Role of the theca and granulosa cells. Gen Comp Endocrinol 47: 440–448

4 Lincoln RF, Scott AP (1984) Sexual maturation in triploid rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol 25: 385–392

5 Nakamura M, Nagahama Y, Iwahashi M, Kojima M (1985) Reproduction of triploid rainbow trout, *Salmo gairdneri*. Proceedings of the 10th Annual Meeting of Japan Society of Comparative Endocrinology p 18

6 Nakamura M, Nagahama Y, Iwahashi M, Kojima M (1987) Ovarian structure and plasma steroid hormones of triploid female rainbow trout. Nippon Suisan Gakkaishi 53: 1105

7 Onozato H (1987) Senshokutai Kougakuteki Shuhouniyoru Ikushu. In: "Kaiyugyo no Seibutugaku" Ed by K Morisawa, K Wada, T Hirano, Japan Science Societies Press, Tokyo, pp 235–251 (in Japanese)

8 Sakai N, Ueda H, Suzuki N, Nagahama Y (1989) Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different developmental stages. Gen Comp Endocrinol 75: 231–240

9 Satoh S, Tomita M,Iwahashi M, Nakamura M (1991) Reproduction of triploid tilapia, *Oreochromis niloticus*. Scientific Reports of Niigata Prefecture Inland Water Fisheries Experimental Station. 16: 73–81 (in Japanese)

10 Solar II, Donaldson EM, Hunter G (1984) Induction of triploidy in rainbow trout (*Salmo gairdneri* Richardson) by heat shock, and investigation of early growth. Aquaculture 42: 57–67

11 Suzuki R, Nakanishi T, Oshiro T (1985) Survival, growth and sterility of induced triploids in the cyprinid loach *Misgurnus anguillicaudatus*. Bull Jap Soc Sci Fish 51: 889–894

12 Thorgaard GH (1983) Chromosome set manipulation and sex control in fish. In "Fish Physiology Vol IXB" Ed by WS Hoar, EM Donaldson, Academic Press, New York, pp 405–434

13 Ueda H, Kagawa H, Nagahama Y (1985) Involvement of gonadotropin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. Gen Comp Endocrinol 59: 24–30

14 Ueda H, Kambegawa A, Nagahama Y (1984) *In vitro* 11-ketotestosterone and 17α,20β-dihydroxy-4-pregnen-3-one production by testicular fragments and isolated sperm of rainbow trout, *Salmo gairdneri*. J Exp Zool 231: 435–439

15 Ueda H, Young G, Crim LW, Kambegawa A, Nagahama Y (1983) 17α,20β-Dihydroxy-4-pregnen-3-one: Plasma levels during sexual maturation and *in vitro* production by the testes of amago salmon (*Oncorhynchus rhodurus*) and rainbow trout (*Salmo gairdneri*). Gen Comp Endocrinol 51: 106–112

16 Ueno K (1985) Sterility and secondary sexual char-

acter of triploid *Gnathopogon elongatus caerulescens*. Fish Genetics and Breeding Science 10: 37–41

17 Wolters WR, Libey GS, Chrisman GL (1982) Effect of triploidy on growth and gonadal development of channel catfish. Trans Am Fish Soc 111: 102–105

18 Young G, Crim LW, Kagawa H, Kambegawa A, Nagahama Y (1983) Plasma 17α,20β-dihydroxy-4-pregnen-3-one levels during sexual maturation of amago salmon (*Oncorhynchus rhodurus*): Correlation with plasma gonadotropin and *in vitro* production by ovarian follicles. Gen Comp Endocrinol 51: 96–105